

Anti-insect defenses of *Achnatherum robustum* (sleepygrass) provided by two *Epichloë* endophyte species

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This is the peer reviewed version of the following article:

Shymanovich, T. and Faeth, S. H. (2018), Anti-insect defenses of *Achnatherum robustum* (sleepygrass) provided by two *Epichloë* endophyte species. *Entomologia Experimentalis et Applicata*, 166(6): 474-482.

which has been published in final form at <https://doi.org/10.1111/eea.12692>. This article may be used for non-commercial purposes in accordance with [Wiley Terms and Conditions for Use of Self-Archived Versions](#).

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Abstract:

Many pooid grasses (Poaceae) harbor *Epichloë* species (Hypocreales), endophytic fungi that often produce toxic alkaloids which may provide anti-insect protection for their hosts. Two natural populations of *Achnatherum robustum* (Vasey) (sleepygrass), in the Lincoln National Forest, Cloudcroft, and Weed (NM, USA), are infected with the endophyte species *Epichloë funkii* (KD Craven & Schardl) JF White and *Epichloë* sp. nov. We tested whether: (1) these endophytes affect survival, growth, and development of the insect herbivore *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae) (fall armyworm), (2) larval diets alter adult fecundity (assessed as number of larvae or eggs produced by females and number of spermatophores that males transfer to females when enclosed in pairs within each feeding group), and (3) infections affect leaf consumption in larval no-choice and choice experiments. Individual larvae were reared on *Epichloë* infected vs. uninfected clipped leaves from the Cloudcroft and Weed population plants. Overall, armyworm survival was not affected when fed infected sleepygrass from either population. However, larvae that fed on Weed-infected plants were smaller and had longer development than larvae that fed on uninfected and Cloudcroft-infected plants. Males fed on Weed-infected leaves had reduced mating success. Interestingly, pupal mass increased when larvae fed on either the infected leaf types. However, heavier females from both infected diets did not lay more eggs than lighter females from uninfected diets. In a no-choice test, larvae on Weed-infected plants diet consumed more leaf biomass than larvae from three other groups. In choice tests, larvae avoided feeding on leaves infected with either of the endophytes relative to uninfected leaves. Thus, the two *Epichloë* may provide direct protection to sleepygrass from insect herbivory by deterrence. The Weed population endophyte may provide stronger indirect protection than the Cloudcroft endophyte by reducing insect fitness or increasing risks of predation and parasitism through delayed development, even though larvae may consume more leaf biomass.

Keywords: insect herbivores | fall armyworm | endophytic alkaloids | indole-diterpenes | deterrence | delayed development | fecundity | *Spodoptera frugiperda* | Lepidoptera | Noctuidae | Poaceae | Hypocreales

Article:

Introduction

Plants have evolved elaborate chemical and physical defense mechanisms against insect herbivores (e.g., Agrawal, 2011; Mithöfer & Maffei, 2016). For chemical defenses, some cool-season, pooid grasses have partnered with fungal symbionts in the genus *Epichloë* (Hypocreales) (Schardl, 2001). Some species of *Epichloë* are asexual and are strictly transmitted vertically by growing into host seeds (previously placed in the genus *Neotyphodium*; Leuchtman et al., 2014) and therefore are expected to be strong mutualists, especially relative to protection against herbivores (e.g., Clay & Schardl, 2002). *Epichloë* endophytes can produce an array of alkaloids within four major classes, including ergot alkaloids, indole-diterpenes, lolines, and peramine, depending on the presence of complex genes, whereas environmental factors may affect alkaloid levels (Schardl et al., 2013). Some ergot alkaloid and indole-diterpene compounds are known to be toxic to mammals. However, *Epichloë* species are also well renowned for producing a variety of alkaloid compounds that may be toxic to, or deter, insect herbivores (Cheplick & Faeth, 2009; Schardl et al., 2013; Panaccione et al., 2014). Direct protection against insect herbivores may be due to insecticidal compounds such as loline alkaloids *N*-formyllooline, *N*-acetylnorlooline, and the ergot alkaloid ergovaline or due to insect-detering alkaloidal compounds such as peramine (Rowan et al., 1986; Potter et al., 2008; Jensen et al., 2009; Popay et al., 2009). Specific fungal alkaloid compounds from all four classes may cause delayed development and reduced mass and fecundity, which may decrease insect fitness and therefore indirectly reduce insect population sizes and densities (Braman et al., 2002; Härrä et al., 2008; Dmitriew & Rowe, 2011; Saari et al., 2014; Vélez et al., 2014).

Achnatherum robustum (Vasey) Barkworth [= *Stipa robusta* (Vasey) Scribn. = *Stipa vaseyi* Scribn.] (Poaceae, Pooideae, Stipeae), or sleepygrass, is a wild grass native to the western and southwestern USA and northern Mexico (Petroski et al., 1992; Jones et al., 2000). As the common name implies, sleepygrass has long been known for its narcotizing and toxic effects on horses and cattle (Bailey, 1903; Marsh & Clawson, 1929). The toxic effects are caused by the ergot alkaloids ergonovine and lysergic acid amide, produced by a fungal endophyte that was originally named *Acremonium* (Petroski et al., 1992) and is currently classified as *Epichloë* (Leuchtman et al., 2014). These compounds cause dizziness, narcotization, weakness, elevated body temperature, frequent urination, diarrhea, and potential death in livestock (Petroski et al., 1992). However, later studies demonstrated that although the infection by *Epichloë* is widespread throughout the range of sleepygrass, the highly toxic nature of sleepygrass is limited to a small fraction of the range of the grass near Cloudcroft, NM, USA (Faeth et al., 2006).

Previously, only one endophyte, *Epichloë funkii* (KD Craven & Schardl) JF White, has been described from this host (Moon et al., 2007). However, there is now molecular genetical and chemical evidence that there is a second endophyte. This latter endophyte is responsible for the

highly toxic effects on livestock (Faeth et al., 2006; Shymanovich et al., 2015). Infection by this endophyte appears restricted to sleepygrass in the Cloudcroft area in the Lincoln National Forest. The endophyte is a new species (it differs in mating type and alkaloid genes from *E. funkii*) and is currently being described (T Shymanovich, M Oberhofer, CA Young, ND Charlton, CL Schardl & SH Faeth, unpubl.). This endophyte (hereafter referred to as *Epichloë* sp. nov.) produces several ergot alkaloids, often at high levels (Faeth et al., 2006), such as chanoclavine I, ergonovine, lysergic acid amide, and one indole-diterpene compound, paspaline (Shymanovich et al., 2015).

Alternatively, the much more widespread endophyte *E. funkii*, based on the alkaloids produced, does not have strong toxic effects on livestock although uninfected grass may be preferred to infected grass in choice tests by cattle (Jones et al., 2000). We have sampled a sleepygrass population near Weed, NM, and found that grasses are infected with *E. funkii*. *Epichloë funkii* from the Weed population can produce chanoclavine I, an ergot alkaloid, and indole-diterpenes such as paspaline, and several terpendoles (E, I, J, C). The peramine gene is present but inactive (Shymanovich et al., 2015).

Correlative and experimental field studies showed that an endophyte from the Cloudcroft population may affect arthropods similar to other *Epichloë* spp. Infection and its associated alkaloids reduce abundances of arthropod predators or parasitoids, negatively affect generalist herbivores, and provide enemy-free space for specialized herbivores that are capable of alkaloid detoxification and sequestering for their own defense (Jani et al., 2010; Faeth & Saari, 2012; Saari et al., 2014). Insect bioassays using the generalist herbivore *Rhopalosiphum padi* (L.) indicate that aphids did not survive on infected plants originating from the Cloudcroft population (Shymanovich et al., 2015). Less is known about the effects of the endophyte from the Weed population, *E. funkii*, on insects. Shymanovich et al. (2015) demonstrated that aphids survive on infected plants originating from the Weed population, but aphid population sizes were not compared to populations on uninfected plants.

Our goal here was to test whether the two endophytes, *E. funkii* and *Epichloë* sp. nov., provide plant defense against generalist herbivores via endophytic allelochemicals. Generalists may lack alkaloid-specific detoxification mechanisms (Faeth & Saari, 2012). We selected fall armyworm moth larvae, *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae), as a generalist insect herbivore. Unlike the sap-feeding hemimetabolous *R. padi*, fall armyworm is a holometabolous, chewing insect herbivore. We tested how infection by the two endophytes, *Epichloë* sp. nov. and *E. funkii*, from two nearby grass populations (Cloudcroft and Weed, respectively) affected larval performance, larval food choice, and adult fecundity.

Materials and methods

Epichloë endophytes

Asexual *Epichloë* (Clavicipitaceae) infections are systemic and completely asymptomatic in the plant host. Hyphae grow in intercellular spaces without damaging cells. The plant host provides habitat, nutrition, and vertical transmission as hyphae grow into developing seeds (e.g., Cheplick & Faeth, 2009; Schardl et al., 2009; Leuchtman et al., 2014; Schirrmann & Leuchtman, 2015).

Distribution of *E. funkii* endophyte is widespread across Arizona, Colorado, and New Mexico (USA) (Faeth et al., 2006; Moon et al., 2007). Based on alkaloid profiles, *Epichloë* sp. nov. is more localized and thus far has only been found near Cloudcroft, NM (Faeth et al., 2006). Both endophyte species are hybrids and appear completely asexual (sexual stages have not been observed) and thus are likely strictly vertically transmitted by hyphae growing into developing seeds (Faeth et al., 2006; Shymanovich et al., 2015). *Epichloë funkii* has *Epichloë elymi* Schardl & Leuchtman. and *Epichloë festucae* Leuchtman, Schardl & MR Siegel as ancestral progenitors (Moon et al., 2007). *Epichloë* sp. nov. is currently being described.

Host grass

Achnatherum robustum is a perennial bunch grass growing on dry plains and hills in open woods or clearings in the southwest USA and northern Mexico above 2 500 m elevation. It is an obligate outcrossing species and reproduces by seed (Faeth et al., 2010). To test the effect of *E. funkii* and *E. sp. nov.* on fall armyworm, we used the second generation plants (F_2) originating from the two natural populations in the Lincoln National Forest, NM. One population was located near Cloudcroft (32°57.5'N, 105°43.1'W), the second population was located near Weed (32°47.7'N, 105°35.7'W) (Faeth et al., 2006). Plants were propagated at the Arboretum at Flagstaff, AZ, USA. We collected seeds from them in 2010 and experimentally removed the endophytes from some of the seeds via heat treatment (4 h soaking in 1.5 ml tube, 20 min in water bath at 55 °C) in 2011 to have similar plant genotypes in the infected and uninfected groups. From these seeds, plants were grown and maintained in a greenhouse. Before our experiments in 2016, we checked the infection status of each plant via immunoassay (Phytoscreen Immunoblot Kit; Agrinostics, GA, USA). Six F_3 plants that originated from the Cloudcroft population [$n(F_1) = 2$, $n(F_2) = 3$] were infected (C+), and three F_3 [$n(F_1) = 2$, $n(F_2) = 3$] were uninfected (C-). Nine F_3 plants that originated from the Weed population [$n(F_1) = 2$, $n(F_2) = 4$] were infected (W+), and two F_3 [$n(F_1) = 1$, $n(F_2) = 2$] were uninfected (W-). As these endophytes are maternally transmitted within host seeds, one F_1 plant corresponds to one fungal genotype.

Insect herbivore

We used fall armyworm larvae and adults for the bioassays. This species is a generalist herbivore but prefers grasses and is widely distributed across the USA including New Mexico and Arizona (EPPO Global Database; Sparks, 1979). Moreover, fall armyworm previously has been used often in grass-endophyte herbivore bioassays (e.g., Hardy et al., 1985; Clay & Cheplick, 1989; Ball et al., 2006; Crawford et al., 2010). Eggs were purchased from the Frontiers Scientific Services (Newark, DE, USA), lot #I_030316sf. When hatched (26 °C, L14:D10), larvae were reared on oat (*Avena sativa* L.) leaf clippings.

Larval performance experiment

The goal of this experiment was to compare larval performance when fed with natural endophytic diets to larval performance on uninfected diets while controlling for any between-population differences in leaf nutrition. For this experiment, individual 2-day-old larvae were enclosed in plastic containers (Plant Con; MP Biomedicals, Solon, OH, USA) and randomly

assigned to one of four diets: C- (n = 12), C+ (n = 20), W- (n = 12), and W+ (n = 20). Larvae were reared in a growth chamber (Adaptis A1000; Controlled Environments, Winnipeg, MB, Canada) under constant conditions (26 °C, L14:D10). Larvae were provided with equal mass of fresh clippings (mixed from the same infection type of plants) ad libitum (Figure S1). To estimate total dry mass of wet leaf material provided, we weighed six wet subsamples from the consecutive feedings for each diet group, air-dried, and recorded dry mass to obtain mean wet to dry mass coefficient, and then, total wet mass was multiplied by these coefficients. Remaining leaves were removed, air-dried, and weighed. The amount of dry biomass consumed was estimated as a difference between dry mass provided to the larvae and how much remained after feeding. Larval mass was recorded on the 5th and 8th days and at pupation. Sex was determined at the pupal stage with a microscope (Bhattacharya et al., 1970). We recorded time to pupation and to adult emergence daily. We also recorded survival if the development cycle was completed and the adult was ready to mate.

Adult fecundity experiment

To determine whether larval diet affected adult fecundity, emerged females and males from the same diet group (C-, n = 5; C+, n = 8; W-, n = 4; W+, n = 7) were randomly placed into single-pair mating cages for 7 days. Adults were provided with 10% honey solution and weighing paper for laying eggs. To estimate female fecundity, eggs were removed every other day and enclosed in Petri dishes for hatching. On the 5th day, neonates and unhatched eggs were fixed with 70% ethanol in Petri dishes, and they were counted from digital photos. To account for larval cannibalism, we added head capsule counts to the total number. Male fecundity was estimated as the number of successful couplings and was assessed by recording the numbers of spermatophores transferred into the bursa copulatrix (Crespo et al., 2010). Briefly, each female moth was mounted, the abdomen was dissected under a microscope, the bursa copulatrix was separated, and the spermatophores were extracted (Figure S2).

Larval feeding choice experiment

To test whether the two endophytes are deterrent to armyworms, 30 single, 3-day-old larvae were enclosed for 48 h in 9 × 9 × 9 cm plastic containers (Plant Con) with wet paper towels and four choices of sleepygrass leaves: uninfected (C-) and infected (C+) from the Cloudcroft population and uninfected (W-) and infected (W+) from the Weed population. The four leaf types were randomly placed on four sides of containers according to labels on a paper towel. Five-day-old larvae were unable to move leaf pieces, so diet placement remained intact before and after the experiment. For each leaf type, we put two pieces of the same size (5 cm) randomly selected from plants within a group (Figure S3). All enclosures were placed into a growth chamber at 26 °C and L14:D10 photoperiod. For each leaf type, the leaf length that was consumed was recorded, and percentage consumed was calculated by dividing by 10 cm (the total length provided) and multiplying by 100%.

Statistical analysis

Fall armyworm survival on various diets for larval, pupal, and adult life stages and for sex was compared with non-parametric Kruskal–Wallis (K–W) rank sum tests. Data for developmental

time to pupation and to adult emergence were non-normally distributed, and transformations did not normalize the data. K–W rank sum tests with sex as a factor showed that development time to pupation and to adult emergence differ for female ($P = 0.08$) and male larvae ($P < 0.0001$). Therefore, we used K–W rank sum tests separately for female and male larvae on the four diets and Bonferroni correction for pairwise comparisons within each group. Two-way ANOVA tests of diet and sex as factors in determining larval and pupal mass showed that sex did not significantly affect development at 5 and 8 days of larval development and at pupation ($P = 0.38$, 0.94 , and 0.09 , respectively). Therefore, we used one-way ANOVA models with only diet as a factor. To normalize data distributions, we took the natural logarithm of larval mass at 8 days and at pupation. The amount of dry plant biomass consumed was ln-transformed and compared with one-way ANOVA. Female fecundity (number of neonates and eggs) on different diets was compared with one-way ANOVA. Male fecundity (number of spermatophores transferred) on different diets was compared with K–W rank sum test and Bonferroni correction for pairwise comparisons. We also used K–W rank sum test and Bonferroni corrections to compare the multiple pairwise test of larval choice of sleepygrass leaf types. All analyses were performed with R i386 3.3.2 software with R Commander and Dunn Test packages (R Development Core Team, 2008).

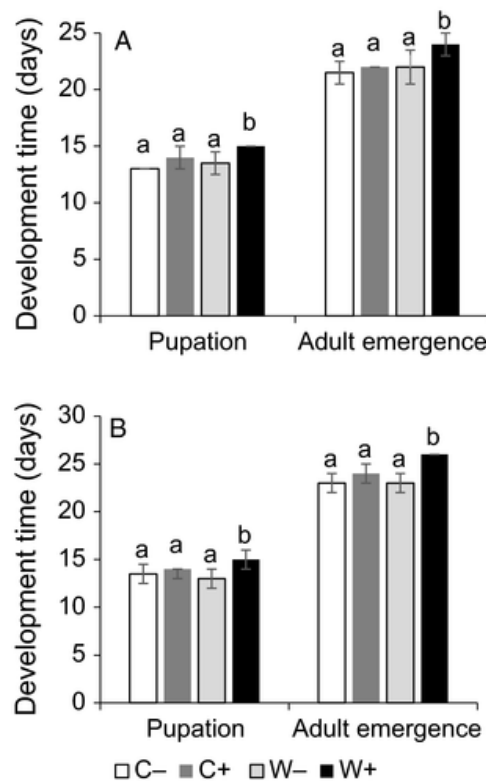


Figure 1. Median (\pm interquartile range) development time (i.e., time to pupation and time to adult emergence) of (A) female and (B) male *Spodoptera frugiperda* larvae reared on one of four *Achnatherum robustum* diets: Cloudcroft population plants uninfected (C–) and endophyte-infected (C+), and Weed population plants uninfected (W–) and infected (W+). Larvae were fed with leaf clippings from greenhouse-grown plants that originated from the two populations. Medians capped with different letters are significantly different (Kruskal–Wallis rank sum with Bonferroni pairwise comparisons: $P < 0.05$).

Results

In the fall armyworm performance experiment, larval survival in uninfected groups was high (83 and 92%). Larvae, pupae, and adults on the four diets (C⁻, C⁺, W⁻, and W⁺) ($P = 0.23$, 0.41 , and 0.52 , respectively) and of both sexes ($P = 0.26$) had similar survival rates (Table S1). However, development time to pupation and to adult emergence for females and for males was delayed when they fed on Weed infected (W⁺) leaf diet compared to larvae feeding on uninfected leaves from both populations (C⁻, W⁻) and on infected leaves from the Cloudcroft population (C⁺) as well (Figure 1, Table S1).

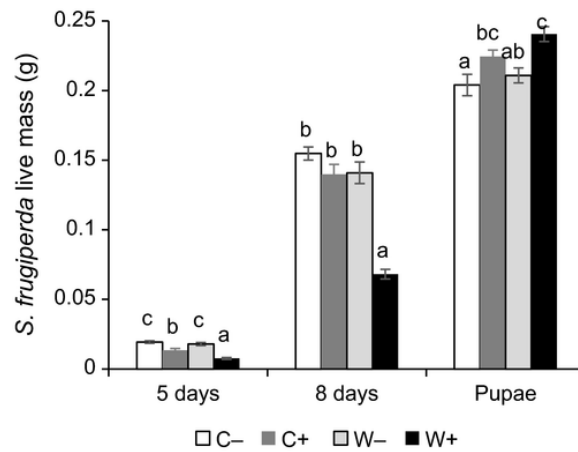


Figure 2. Mean (\pm SE) live mass (g) of 5- and 8-day-old *Spodoptera frugiperda* larvae and pupae combined for both sexes when reared on one of four *Achnatherum robustum* diets: Cloudcroft population plants uninfected (C⁻) and endophyte-infected (C⁺) and Weed population plants uninfected (W⁻) and infected (W⁺). Means capped with different letters are significantly different (one-way ANOVA: $P < 0.05$; Table S1).

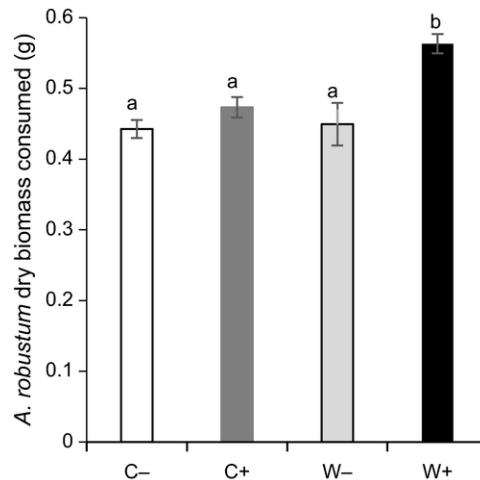


Figure 3. Mean (\pm SE) dry biomass (g) consumed by *Spodoptera frugiperda* larvae reared on one of four *Achnatherum robustum* diets: Cloudcroft population plants uninfected (C⁻) and endophyte-infected (C⁺) and Weed population plants uninfected (W⁻) and infected (W⁺). Means capped with different letters are significantly different (one-way ANOVA: $P < 0.01$; Table S1).

Diets had contrasting effects on larval and pupal mass. At 5 days, live larval mass was reduced in both groups when feeding on infected leaf types from either population (C⁺ and W⁺) compared to their uninfected counterparts (Figure 2, Table S1). At 5 days, larvae on W⁺ diet were the smallest of all diet groups. At 8 days, only larvae feeding on the W⁺ diet had reduced mass in comparison to the three other groups. However, pupal mass increased when feeding on infected

leaves compared to uninfected sleepygrass leaves from the same population (Figure 2). Larvae on W+ diet consumed more dry plant biomass than larvae from three other diet groups (Figure 3, Table S1).

For the first 7 days of oviposition, there was no difference in female fecundity, as assessed by the number of neonates and unhatched eggs, for moths from the various diet groups ($P = 0.25$) (Figure 4A). However, male fecundity, as assessed by the number of spermatophores transferred, was reduced for W+ diet group (Figure 4B, Table S1).

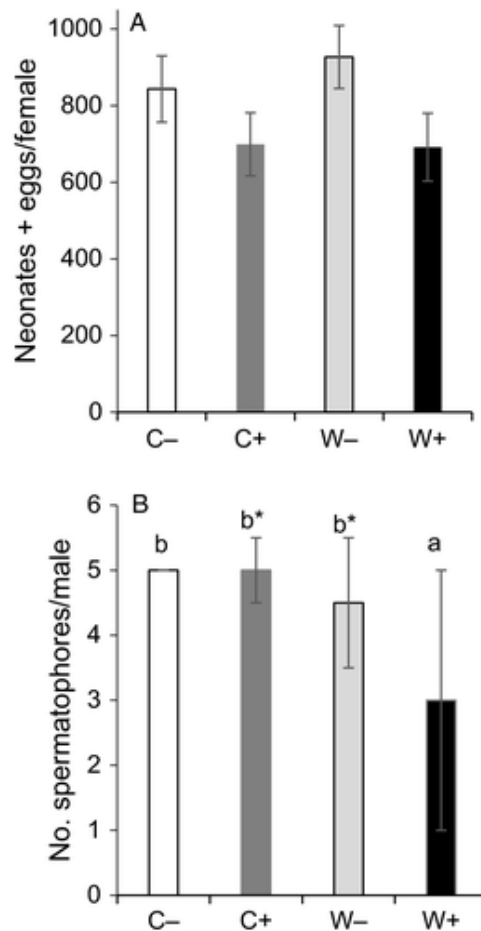


Figure 4. (A) Mean (\pm SE) total number of neonates + eggs per female and (B) median (\pm interquartile range) number of spermatophores transferred per male (from a single-pair mating) as indicators of female and male fitness for *Spodoptera frugiperda* reared on one of four *Achnatherum robustum* diets: Cloudcroft population plants uninfected (C-) and endophyte-infected (C+), and Weed population plants uninfected (W-) and infected (W+). Larvae were fed leaf clippings from greenhouse-grown plants that originated from the two populations ($n = 23$ in total). Different letters capping columns indicate significant difference [ANOVA I (females), Kruskal–Wallis rank sum with Bonferroni pairwise comparisons (males): $P < 0.05$]. Asterisks indicate borderline difference between W+ and W- ($P = 0.083$) and between W+ and C+ ($P = 0.089$) groups.

In the choice experiment, larvae preferred to eat uninfected leaves from the Cloudcroft population plants relative to the other leaf types (Figure 5, Table S1). Their second choice was uninfected leaves from the Weed population plants. Larvae generally avoided feeding on infected leaf material from either of the populations.

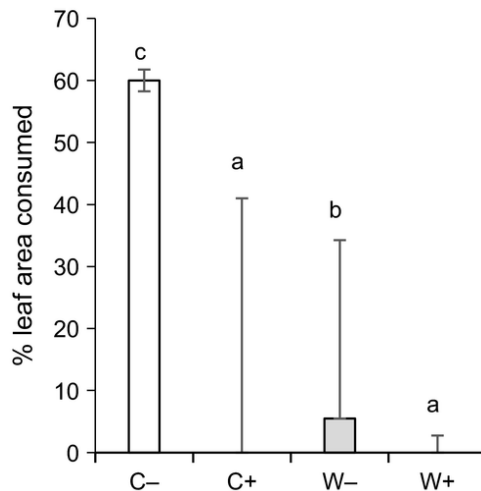


Figure 5. Median (\pm interquartile range) *Spodoptera frugiperda* larval choice of *Achnatherum robustum* leaf types from Cloudcroft population plants uninfected (C-) and endophyte-infected (C+), and Weed population plants uninfected (W-) and infected (W+) after 48 h. Medians capped with different letters are significantly different (Kruskal–Wallis rank sum with Bonferroni pairwise comparisons: $P < 0.01$).

Discussion

Even though fall armyworms are intensively used for herbivory research, there are no previous studies utilizing sleepygrass. In our project, we compared larval performances when fed with endophyte-infected plants vs. endophyte-free plants from the same population. We observed that fall armyworms fed on *Epichloë*-free sleepygrass survive and develop relatively well. In Braman et al. (2002), fall armyworm larval survival on uninfected *Festuca rubra* L., *Lolium perenne* L., and *Festuca arundinacea* Schreb. plants ranged from 35 to 79% which is lower or similar to our study results (83 and 92%). Mean pupal mass in Braman et al. (2002) ranged from 188 to 239 mg, so our results for this parameter (204 and 211 mg) are intermediate. However, we should caution these comparisons as their study used lower temperature (24 °C).

Our study provides additional evidence that the two endophytes in sleepygrass may be effective deterrents against, or have negative effects on, generalist insect herbivores. Both endophytes, *E. funkii* and *Epichloë* sp. nov., from the Weed and the Cloudcroft populations, respectively, had deterring effects on feeding by fall armyworm, a generalist chewing herbivore. However, we did not find that either of the endophytes had direct negative effects on fall armyworm in terms of survival, unlike their effects on aphid survival (Shymanovich et al., 2015). In previous experiments, aphid survival was very low on Cloudcroft plants infected with *Epichloë* sp. nov. Shymanovich et al. (2015) surmised that the alkaloid ergonovine in Cloudcroft plants was responsible for aphid mortality because this alkaloid reduced aphid numbers in a direct-feeding experiment. Thus, ergonovine may affect fall armyworm larvae differently than aphids. Clay & Cheplick (1989) found that ergonovine may reduce fall armyworm larval mass but had no effect on leaf consumption. Our results correspond to their study. Larval mass at 5 days was reduced, but leaf consumption in the no-choice experiment was similar to larvae on uninfected plants.

Another difference from Shymanovich et al. (2015) is that fall armyworm larvae in our study were fed excised leaf clippings whereas in their study, aphids fed directly on live plants.

Alkaloid levels might be altered by clipping or by aphid feeding. For example, Fuchs et al. (2016) reported that leaf clipping increased the concentration of the alkaloid lolitrem B but did not affect ergovaline and peramine concentrations in *L. perenne* tissues infected with *E. festucae* var. *lolii*. Aphid feeding in their study did not affect alkaloid levels. However, we would expect that larval survival would decrease if clipping increased alkaloids harmful to fall armyworm, and clearly, larval survival was not affected by infection by either endophyte in clipped leaves. Hypothetically, leaf chewing by fall armyworms may affect alkaloid levels more than does artificial clipping. For example, Fuchs et al. (2016) showed that locusts chewing on infected perennial ryegrass increase alkaloids more than leaf clipping.

Although we found no negative effects on larval survival, both endophytes had several other negative effects on fall armyworms that may reduce their fitness or increase risks to predation and parasitism. In ideal conditions, insect larvae have rapid growth, earlier transitions to the next stages, and often they are larger (Day & Rowe, 2002). Larvae feeding on a restricted or toxic food source often exhibits reduced fitness as adult (de Sassi et al., 2006; Dmitriew & Rowe, 2011). In our experiment, larvae that fed on infected plants from the Cloudcroft and Weed populations had reduced mass at 5 days old, but at 8 days old, only the larvae that fed on Weed-infected plants had reduced mass, in comparison to larvae that fed on uninfected plants. Delayed development was also more evident for larvae on the W+ diet than on the C+ diet. Most likely, these effects were due to initially strong feeding deterrence from C+ and W+ infected plant material, but malnutrition may also affect hormonal functions (Slama, 1978). Moreover, some plant and fungal alkaloids are known to affect insect juvenile hormone biosynthesis and therefore developmental stages (Hoffmann & Lorenz, 1998). To the best of our knowledge, the effects of *Epichloë* alkaloids on biosynthesis and function of insect hormones have yet to be tested. Delayed development of insects, especially at the late larval and pupal stages, may increase mortality risks from predators and parasitoids (Price et al., 1980; Hawkins et al., 1997).

Pupal mass increased for larvae feeding on C+ and W+ endophyte-infected plant material compared to their uninfected counterparts. A similar effect was described in the study by Braman et al. (2002), in which fall armyworm pupal mass was increased when larvae fed on *F. rubra* infected with *E. festucae* compared to those that fed on uninfected plants. This result might be construed as evidence of a positive effect of infected plants on armyworm fitness that partially counteracts the negative effects of delayed development. Heavier females usually produce more eggs (Vélez et al., 2014). However, this increase in pupal mass did not result in increased female fecundity in our experiment. Moreover, males reared on Weed-infected plants had fewer successful couplings. We are unsure of the reason for a gain in pupal mass when larvae fed upon infected plants. One possibility is that larvae consumed more plant material on W+ plants to compensate for more toxic or less nutritious food, thus leading to increased pupal mass. Another explanation may be that fall armyworms, like some other lepidopteran species, sequester alkaloids, and in some cases, this may increase mass (Opitz & Müller, 2009). Endocrine hormone imbalance effects may also be a factor in mass differences (Hoffmann & Lorenz, 1998; Gäde & Goldsworthy, 2003).

Both endophytes had strong deterring effects on fall armyworm larvae, given the strong preference for uninfected plants from the respective populations. Although this difference within populations may be explained by alkaloids produced by the respective endophytes, there is also a

clear difference in preference between populations. When given the choice, 2- to 4-day-old larvae consumed far less of uninfected leaves from the Weed than from the Cloudcroft population. These differences may be due to variation in leaf nutrition, water content, or physical defenses as plants from the Weed population appear less nutritious and have tougher leaves. Sleepygrass from the Weed population is likely adapted to an environment that has relatively less precipitation and poorer soils (Jani et al., 2010; Jia et al., 2015) than the Cloudcroft site, even though all plants for this experiment were treated similarly in the greenhouse.

Although we found deterring or toxic effects of both endophytes, our results differ from those of Shymanovich et al. (2015). Shymanovich et al. (2015) found that *Epichloë* sp. nov.-infected plants from Cloudcroft had stronger negative effects on aphids than *E. funkii*-infected plants from Weed. Instead, the reverse occurs for fall armyworm. Larvae fed with leaves infected with *E. funkii* were smaller at 5 and at 8 days, and their development time to pupation and to adult emergence was longer than for larvae fed on leaves infected with *Epichloë* sp. nov. The longer lasting deterring effects of *E. funkii*-infected plants might be responsible as larval mass at 8 days was reduced only in W+ group. Furthermore, male moths on W+ diet had fewer couplings than males on C+ diet.

It is difficult to ascertain what specific alkaloids or other chemical or physiological changes in infected plants from Cloudcroft and Weed are responsible for delayed developmental times and deterrence of fall armyworm on infected plants within each population. It is possible that differences in indole-diterpene alkaloid levels may partially account for the more toxic and deterring effects on fall armyworm of infected Weed plants compared to infected Cloudcroft plants. *Epichloë funkii* produces not only paspaline but also terpendoles E, I, J, and C, whereas *Epichloë* sp. nov. produces only paspaline. These additional alkaloids may increase its deterrence and toxicity to fall armyworms. However, it is important to note that specific alkaloids (e.g., paspaline) can be produced in widely varying amounts, from trace levels to enormously high levels, depending on the endophyte species and strain, the host plant, and environmental conditions (Schardl et al., 2013; Panaccione et al., 2014). For example, different plants and plant tissues may have varying alkaloid concentrations due to host nutrition and age, seasonal changes, insect grazing, or hyphal density (e.g., Hunt et al., 2005; Rasmussen et al., 2007; Helander et al., 2016). In addition, various intermediate products (e.g., chanoclavine I) in complex pathways may or may not be biologically active. Finally, each class of alkaloids and each alkaloid within a class have a specific and unique biological activity. For example, insect species may have individual susceptibility to various alkaloidal compounds (Siegel et al., 1990; Crawford et al., 2010; Schardl et al., 2013). Further complicating the picture is that endophytic alkaloids could be sequestered by the insect herbivore and used as defense against their own natural enemies (Saari et al., 2014).

In conclusion, *Epichloë* species from two sleepygrass populations provide anti-herbivore protection from fall armyworms mainly due to deterrence effects but also likely due to reduced insect fitness or higher predation and parasitism risks, especially *E. funkii*. How these effects translate into reduced herbivory or reduced insect herbivore populations in the field on infected plants, where other complicating factors may prevail, is yet to be determined.

Acknowledgements

This research was supported by NSF Grant DEB 0917741 to S.H.F. We gratefully thank Dr. Scott Richter (UNCG) for assistance with statistical analyses.

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